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Oxytetracycline and thermal marking of alewife (Alosa pseudoharengus) otoliths

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OXYTETRACYCLINE AND THERMAL MARKING OF ALEWIFE (ALOSA PSEUDOHARENGUS) OTOLITHS

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I dedicate this thesis to my Uncle Bill Luell.

Retired Senior Chief Petty Officer, USCG.

Semper Paratus

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ABSTRACT

OXYTETRACYCLINE AND THERMAL MARKING OF

ALEWIFE (*ALOSA PSEUDOHARENGUS*) OTOLITHS

By Steven M. Luell

University of New Hampshire, September 2016

Alewives (*Alosa pseudoharengus*) are anadromous clupeids native to the east coast of North America. With their population in decline, there is increasing interest in releasing hatchery-reared alewives for stock enhancement. As a result, techniques are needed to produce long-lasting marks to identify stocked fish. The objective of this investigation was to evaluate the efficacy of marking alewives using oxytetracycline immersion and thermal marking techniques. Oxytetracycline marking trials consisted of immersing alewife larvae in three concentrations of oxytetracycline (200, 400, and 600 mg/L) for two durations (4 and 6 hours). Sagittal otoliths were removed and examined for marks with an epifluorescence microscope after 158, 232, and 350 DPH in October 2015, January 2016 and May 2016. Exposure to higher concentrations and longer durations in the immersion bath resulted in a higher percentage of fish with visible marks. Oxytetracycline marks were present on 100% of the otoliths sampled from the 6-hour immersion in 600 mg/L. Alewife larvae and juveniles were exposed to various temperature change regimes to produce thermal marks on the sagittal otoliths. All of the juveniles survived the thermal marking but the larvae experienced 100% mortality during the marking procedure. Otoliths were examined for thermal marks after 142, 271, and 349 DPH in

October 2015, February 2016 and May 2016. Exposure to warm water temperatures for 24 hours created wider translucent increments between the opaque bands on the otoliths. There did not appear to be a difference in the lightness of the increments or darkness of the bands between the 25°C: 18°C or 28°C: 16°C treatments. The results of this study indicate that the optimal marking procedure for mass-marking alewives for stock enhancement is immersing larvae in 600 mg oxytetracycline/L for 6 hours.

CHAPTER 1

INTRODUCTION

Life History

Alewives (*Alosa pseudoharengus*) and blueback herring (*Alosa aestivalis*), collectively known as river herring, are small clupeids native to the east coast of North America. Alewives are often distinguished from blueback herring by having a larger eye diameter, greater body depth and lighter perotineal pigmentation (Loesch 1987). Both species are sympatric over much of their ranges. Alewives range from the Gulf of St. Lawrence to South Carolina and blueback herring are distributed from Nova Scotia to the St. John's River in Florida (Collette and Klein-MacPhee 2002; McBride et al. 2010). Some populations of river herring have become residents in freshwater portions of coastal rivers and bays (Foerster and Goodbred 1978; Limburg et al. 2001) or landlocked in large lakes. Landlocked populations of alewives have had a detrimental effect on native zooplankton and fish communities in the Lake Champlain and the Laurentian Great Lakes (Wells 1970; Bunnell et al. 2006; Madenjian et al. 2008; Marsden and Hauser 2009).

River herring are anadromous and migrate into freshwater systems in the spring to spawn. It is presumed that both species return to their natal streams to spawn but some may stray into nearby rivers and streams (Loesch 1987). River herring are iteroparous and spawn several times during their lives (Collette and Klein-MacPhee 2002). Alewives typically begin spawning about 3 to 4 weeks before blueback herring though there is overlap in their spawning seasons (Jones et al. 1978). Spawning is influenced by water temperature, and the timing of the spawning runs

progresses chronologically from south to north throughout their ranges. Alewives begin spawning in water temperatures of 5-10°C, and blueback herring begin spawning between 10 and 15°C (Loesch 1987; Collette and Klein-MacPhee 2002). Both species spawn primarily at night. Blueback herring prefer to spawn over hard substrate in swift currents (Loesch 1987). Alewives spawn in slow flowing waters including coves, oxbows, small tributary streams, ponds and flooded swamps (Loesch 1987; Walsh et al. 2005; Mather et al. 2012). They have also been observed spawning in ponds behind barrier beaches with an open connection to the ocean (Mullen et al. 1986; Collette and Klein-MacPhee 2002). Alewives in river systems blocked by obstructions such as dams or culverts will spawn in deep pools and eddies (Loesch 1987). After spawning, adults return to the ocean though some may remain in the estuary for a period of time, possibly to rest and/or adapt to higher salinity prior to entering the ocean (Mather et al. 2012).

The incubation time of river herring eggs is influenced by temperature. Alewife eggs hatch in 2.1 days at 28.9°C and 15 days at 7.2°C (Jones et al. 1978). Larvae are about 2.5-5.6 mm total length (TL) when they hatch and begin feeding on zooplankton after the yolk-sac is absorbed about 3-5 days post-hatch (DPH; Jones et al. 1978). The metamorphosis from larval to juvenile morphology begins at about 20 mm TL (Mullen et al. 1986). Juveniles begin to migrate to the ocean in the fall. This downstream migration is triggered by decreasing water temperatures, increased stream flow and precipitation (Jones et al. 1978; Gahagan et al. 2010). Some juvenile alewives remain in deep waters of the estuary during the winter before migrating into the ocean in early spring (Hildebrand and Schroeder 1928).

Alewives congregate in large schools numbering in the tens of thousands. During spring, river herring are distributed along the Middle Atlantic Bight before moving north to Nantucket Shoals, Georges Bank and the Gulf of Maine in summer and early fall (Neves 1981; Able and

Fahay 2010). As the temperatures decrease in autumn, they migrate south to the Middle Atlantic Bight for the winter (Neves 1981; Able and Fahay 2010). Alewives exhibit negative phototaxis and are found deeper in the water column than blueback herring during the day (Neves 1981; Loesch 1987; Jessop 1990). This vertical separation between the species may reduce interspecific competition (Loesch 1987). Alewives exhibit a diel vertical migration ascending to surface waters to feed at night (Jessop 1990). Alewives consume a large variety of zooplankton such as calanoid copepods, cladocerans, amphipods, mysids and decapod larvae (Collette and Klein-MacPhee 2002). In the ocean, adults also feed on fish eggs and larvae, as well as small juveniles of several species such as Atlantic herring (*Clupea harengus*), cunner (*Tautogolabrus adspersus*), sand lance (*Ammodytes* spp.), American eel (*Anguilla rostrata*) and even their own species (Collette and Klein-MacPhee 2002).

Population Decline

The populations of many anadromous species in North America have experienced a severe decline in abundance throughout much of their native ranges since European settlement. In 2006, the National Marine Fisheries Service (NMFS) listed river herring as a "species of concern" in the United States. They were also considered to be a candidate for listing as threatened or endangered under the U.S. Endangered Species Act but ultimately were not listed (NOAA 2013). The exact causes of the decline of the river herring population are unknown, but they may be due to a multitude of factors.

One factor that has affected populations of anadromous fish is the loss of spawning habitat due to barriers blocking migratory routes. Industrialization was once dependent on rivers for energy, and dams were constructed in many waterways. The U.S. Army Corps of Engineers'

National Inventory of Dams characterizes about 22,000 dams along the east coast of the United States, though there are substantially more that were not counted because they were smaller than the criteria for the inventory (Graf 1999). In addition to blocking fish passage, dams also effect the ecology of rivers by causing alterations in water temperatures, retaining sediments and nutrients, and changing the structure of resident freshwater fish communities (Limburg and Waldman 2009). Turbines from hydroelectric dams also pose a risk for migrating fish. Current restoration efforts have included dam removal and the construction of fish ladders (Franklin et al. 2012). Alewives begin spawning in newly reopened spawning habitats in about 3-5 years (Pardue 1983; Burdick and Hightower 2006). Culverts have also been constructed at locations where roads cross freshwater streams and salt marsh creeks. Fish passage historically was not considered in the design of many culverts (Eberhardt et al. 2011). Larger culverts that do not alter water velocity have been recommended to maintain hydrology and allow fish passage in salt marshes (Eberhardt et al. 2011). Habitat alteration such as dam construction increases the likelihood of hybridization between the two species (Hasselman et al. 2014). Despite the spatial and temporal differences in spawning preferences, hybridization between the two species has become common in some rivers (McBride 2013; McBride et al. 2014).

Water pollution from commercial and industrial activities has greatly reduced the spawning runs of anadromous fishes in many river systems. Due to poor water quality, many rivers became anoxic for several months out of the year blocking migratory fish passage (Weisburg et al. 1996; Limburg and Waldman 2009). Since the Clean Water Act of 1972 mandated the treatment of municipal and industrial wastewaters, many river systems have recovered considerably, though toxic substances remain in the sediments and biota (Lichter et al. 2006). Urban sprawl in the late 20th century has increased the amount of impervious surfaces in

many watersheds, leading to an increase in the amount of excess nutrients and other forms of non-point source pollution entering the waterways as runoff (Limburg and Waldman 2009).

River herring are commonly caught as incidental bycatch in the Atlantic herring and Atlantic mackerel (*Scomber scombrus*) commercial fisheries (Bethoney et al. 2014; Turner et al. 2015). Bycatches of river herring in the Atlantic herring fishery are highest in January-April and September-December when commercial fishing efforts are primarily off southern New England and the northern Mid-Atlantic Bight (Cournane et al. 2013). This is detrimental to river herring populations as the bycatch rates are highest as adults are preparing to migrate to their spawning habitats during the spring, and when juveniles migrate from their freshwater nurseries into the ocean in the fall.

River herring spawning runs greatly benefit freshwater ecosystems by contributing nutrients from the marine environment into freshwater food webs. The marine-derived nutrients enter the freshwater ecosystem from the adult herring that die during the spawning run, the eggs spawned, and through excretion from the surviving herring that return to the ocean (Post and Walters 2009). The nutrients are quickly incorporated into the freshwater food web and are found at the greatest levels in all of the trophic levels in the stream during the spawning run (Walters et al. 2009).

Many species are altering their distribution in response to climate change and increases in water temperature have influenced the timing of fish migrations (Limburg and Waldman 2009). Ellis and Volkoun (2009) reported that alewife spawning runs in southern New England occur about 12 days earlier on average than runs in the 1970s. In the Androscoggin River in Maine, the alewife spawning run occurred 1.2 days earlier on average between 1983 and 2001

(Huntington et al. 2003). Increasing seasonal water temperatures may lead to a northward shift in the distribution of river herring and changes in the timing of the spawning runs.

Species Importance

Historically, river herring were economically important for coastal communities. They were harvested during their spawning runs for human consumption and for use as bait and fertilizer. Alewives are still commonly used in the spring as bait for the American lobster (*Homarus americanus*) fishery in Maine. Unsustainable harvest rates have compromised many anadromous fish populations including river herring (Schmidt et al. 2003; Limburg and Waldman 2009). As a result, many states have issued moratoria prohibiting the harvest of river herring including Massachusetts, Rhode Island and Connecticut.

Alewives at all life stages are forage for a variety of species in the freshwater and marine environments including American eel, largemouth bass (*Micropterus salmoides*), yellow perch (*Perca flavescens*), bluefish (*Pomatomous saltatrix*), weakfish (*Cynoscion regalis*), striped bass (*Morone saxatilis*) and Atlantic cod (*Gadus morhua*; Loesch 1987; Yako et al. 2000; Ames and Lichter 2013). Clupeids are a major component in the diet of striped bass (Walter et al. 2003). The recent increase in abundance of striped bass may further contribute to the decline of river herring populations (Hartman 2003; Walter et al. 2003; Schultz et al. 2009). The presence of other piscivores such as white perch (*Morone americana*) may also affect the success of restoration efforts in some lakes or estuaries (Moring and Mink 2002). Other predators of alewives include common snapping turtles (*Chelydra serpentina*), northern water snakes (*Nerodia sipedon*), American minks (*Neovison vison*), herring gulls (*Larus argentatus*) and double-crested cormorants (*Phalacrocorax auritus*; Loesch 1987; Dalton et al. 2009).

Marking Techniques

Releasing hatchery-reared fish for stock enhancement has become an important tool in fisheries management. Evaluating the contribution of the stocked fish to the wild population can be complex due to the variability in natural spawning and difficulty in identifying stocked and wild fish (Logsdon et al. 2004). Conventional tagging methods such as Passive Integrated Transponder (PIT) tags, T-bar tags and fin clipping, are not feasible because of the small size of the larvae and the large amounts of fish being stocked. As a result, techniques are needed to produce long-lasting marks on hatchery-reared fish. Marking larvae using fluorescent chemicals has been shown to be a viable method.

Fluorescent chemicals produce marks on the otoliths, scales, fin rays and other bony structures. The chemicals enter the bloodstream and are rapidly incorporated into the calcified structures by binding to calcium ions during mineralization (Frost 1968; Campana 1999). The marks are permanently retained on the internal structures and are found in the growth increment that was being formed when the fish was exposed to the chemical (Campana 2001). Otoliths are commonly examined to detect fluorescent marks.

Otoliths are paired structures in the inner ears of teleosts that function in sound reception and maintaining equilibrium (Campana 1999; Popper and Lu 2000). Teleosts have three pairs of otoliths: sagittae, asterisci and lapilli. Sagittae and lapilli are primarily composed of aragonite crystals and asterisci are made up of vaterite crystals (Campana 1999; Popper et al. 2005). Sagittal otoliths are the largest of the three pairs and are commonly used for biological and taxonomic studies (Gauldie and Nelson 1990). The shapes of the otoliths are species-specific and variation can be found geographically within a species (Lombarte and Lleonart 1993; Galley et al. 2006). Otoliths accrete layers of crystalline calcium carbonate continuously throughout

their lives forming daily growth increments (Brothers et al. 1976; Gauldie and Nelson 1990; Morales-Nin 2000). Due to variations in growth rate throughout the year, annual growth increments also form (Labay and Lauer 2006; Pilling et al. 2007). Unlike scales, fin rays and bone, otoliths are not susceptible to resorption (Campana 1999). One disadvantage of otolith marking is that the fish must be sacrificed to detect the mark.

Fluorescent chemicals can be administered to the fish by immersion, injection or feeding. Injecting individual fish is not a feasible method for marking large amounts of fish and feeding techniques may take a week or more to produce a detectable mark (Pedersen and Carlsen 1991). Feeding techniques can also be inconsistent because not all of the fish feed at the same rate (Pedersen and Carlsen 1991; Honeyfield et al. 2006). Immersing the fish in a chemical bath allows a large amount of fish to be marked in several hours (Baer and Rösch 2008; Stewart and Long 2011). Immersion is the most viable technique for marking large quantities of hatchery-reared fish with fluorescent chemicals.

Several fluorescent chemicals are used to mark fish, including alizarin compounds, calcein and tetracycline antibiotics. Alizarin compounds are effective in producing detectable marks but alizarin complexone significantly reduces the growth rates of larvae by slowing the absorption of the yolk-sac (Baer and Rösch 2008; Meyer et al. 2012). Calcein marks can be detected externally by examining the scales with fluorescent light but rapidly degrade and become undetectable when exposed to sunlight (Hill and Quesada 2010). Immersion of larvae in tetracycline antibiotics, such as oxytetracycline, produces long-lasting marks on the otoliths that can be viewed with epifluorescence microscopy. Oxytetracycline has been used to mark multiple species for stock enhancement including striped bass, walleye (*Sander vitreus*), black crappie (*Pomoxis nigromaculatus*), channel catfish (*Ictalurus punctatus*) and red drum

(*Sciaenops ocellatus*; Reinert et al. 1998; Isermann et al. 2002; Jenkins et al. 2002; Logsdon et al. 2004; Stewart and Long 2011).

Oxytetracycline immersion has also been found to produce detectable otolith marks on alosines such as Allis shad (*Alosa alosa*) and American shad (*Alosa sapidissima*; Hendricks et al. 1991; Lochet et al. 2009). This technique has been used to mark American shad to evaluate natal homing and the contribution of hatchery-reared fish to wild populations. Oxytetracycline marks were detected on 73-98% of the returning American shad sampled between 1995 and 2000 in the Lehigh River in Pennsylvania (Hendricks et al. 2002). In the James River in Virginia, catch rates of American shad increased in 2000 and 2001 due to an influx of sexually mature hatchery-reared shad. Oxytetracycline marks were detected on over 40% of the harvested shad (Olney et al. 2003).

American shad stocking programs have been established along the east coast. The Pennsylvania Fish and Boat Commission stocked over 100 million larvae into the Susquehanna River between 1985 and 1994. Shad returns increased from 1,500 to over 100,000 by 1997 (PFBC 2000). Initially, 90% of the returning shad were hatchery-marked but naturally reproducing shad dominated the spawning runs in the late 1990s (PFBC 2000). Migrating shad were captured and transported by truck to spawning habitats upriver until fish lifts and passageways were constructed at the hydroelectric dams in the lower Susquehanna River in the 1990s. Shad returns decreased during the 2000s due to issues with fish passage (SRAFRC 2012). In recent years, capturing and transporting shad to spawning areas upriver has resumed. Stocking efforts have also continued by marking and releasing about 10-15 million shad larvae annually in the Susquehanna River (SRAFRC 2012). The success of stock enhancement for

restoring anadromous fish populations is dependent on efficient fish passage to suitable spawning habitats.

Another method for mass-marking hatchery-reared fish is thermal marking. By altering the water temperature, unique patterns of light and dark bands can be produced on the otolith (Volk et al. 1999). Dense bands of otolin, a keratin-like protein, form in colder temperatures and higher temperatures form lighter, wider daily increments composed of crystalline calcium carbonate between the bands (Letcher and Terrick 1998). While thermal marking is commonly used to mass-mark hatchery-reared salmonids, little work has been done marking warm water species (Song et al. 2008).

The purpose of this study was to evaluate the efficacy of using oxytetracycline immersion and thermal marking to create long lasting marks on the sagittal otoliths of hatchery-reared alewives. The results from this research will be used to identify the most viable methods to mass-mark alewives for stock enhancement.

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CHAPTER 2

OXYTETRACYCLINE AND THERMAL MARKING OF ALEWIFE (*ALOSA PSEUDOHARENGUS*) OTOLITHS

INTRODUCTION

Alewives (*Alosa pseudoharengus*) and blueback herring (*Alosa aestivalis*), collectively referred to as river herring, are small iteroparous clupeids native to the east coast of North America. Alewives are often distinguished from blueback herring by having a larger eye diameter, greater body depth and lighter perotineal pigmentation (Loesch 1987). Both species are sympatric over much of their ranges. Alewives range from the Gulf of St. Lawrence to South Carolina and blueback herring are distributed from Nova Scotia to the St. John's River in Florida (Collette and Klein-MacPhee 2002; McBride et al. 2010).

River herring are anadromous and migrate into freshwater systems in the spring to spawn. It is presumed that both species return to their natal streams to spawn but some may stray into nearby rivers and streams (Loesch 1987). Alewives typically begin spawning about 3 to 4 weeks before blueback herring though there is overlap in their spawning seasons (Jones et al. 1978). Spawning is influenced by water temperature, and the timing of the spawning runs progresses chronologically from south to north throughout their ranges. Alewives begin spawning in water temperatures of 5-10°C, and blueback herring begin spawning between 10 and 15°C (Loesch 1987; Collette and Klein-MacPhee 2002). Both species spawn primarily at night. Blueback herring prefer to spawn over hard substrate in swift currents (Loesch 1987). Alewives spawn in

slow flowing waters including coves, oxbows, small tributary streams, ponds and flooded swamps (Loesch 1987; Walsh et al. 2005; Mather et al. 2012). They have also been observed spawning in ponds behind barrier beaches with an open connection to the ocean (Mullen et al. 1986; Collette and Klein-MacPhee 2002). Alewives in river systems blocked by obstructions such as dams or culverts will spawn in deep pools and eddies (Loesch 1987). After spawning, adults return to the ocean though some may remain in the estuary for a period of time, possibly to rest and/or adapt to higher salinity prior to entering the ocean (Mather et al. 2012).

The populations of many anadromous species in North America have experienced a severe decline in abundance throughout much of their native ranges since European settlement. A multitude of factors may have contributed to the decline of river herring populations including the loss of spawning habitat due to barriers blocking migratory routes, water pollution, overfishing, bycatch in commercial fisheries, predation by increasing stocks of striped bass (*Morone saxatilis*) and climate change (Schmidt et al. 2003; Limburg and Waldman 2009). In 2006, the National Marine Fisheries Service (NMFS) listed river herring as a "species of concern" in the United States. They were also considered to be a candidate for listing as threatened or endangered under the U.S. Endangered Species Act but ultimately were not listed (NOAA 2013). Many states have issued moratoria prohibiting the harvest of river herring including Massachusetts, Rhode Island and Connecticut.

Historically, river herring were economically important for coastal communities. They were harvested during their spawning runs for human consumption and for use as bait and fertilizer. Alewives are still commonly used in the spring as bait for the American lobster (*Homarus americanus*) fishery in Maine. Alewives at all life stages are forage for a variety of species in the freshwater and marine environments (Loesch 1987; Yako et al. 2000; Ames and

Lichter 2013). River herring spawning runs greatly benefit freshwater ecosystems by contributing nutrients from the marine environment into freshwater food webs (Post and Walters 2009; Walters et al. 2009).

Releasing hatchery-reared fish for stock enhancement has become an important tool in fisheries management (Hendricks et al. 2002; Olney et al. 2003). Evaluating the contribution of the stocked fish to the wild population can be complex due to the variability in natural spawning and difficulty in identifying stocked and wild fish (Logsdon et al. 2004). Conventional tagging methods such as Passive Integrated Transponder (PIT) tags, T-bar tags and fin clipping, are not feasible because of the small size of the larvae and the large amounts of fish being stocked. As a result, techniques are needed to produce permanent, long-lasting marks on hatchery-reared fish.

The purpose of this study was to evaluate the efficacy of using oxytetracycline immersion and thermal marking to create long lasting marks on the sagittal otoliths of hatchery-reared alewives. Six treatments of oxytetracycline immersion were evaluated to determine if exposure to higher concentrations for longer durations produced a higher percentage of fish with visible marks. The objectives of the thermal marking experiments were to determine if exposure to warmer temperatures produced wider translucent increments between the opaque bands and if greater water temperature changes produced more distinct marks. The results from this research will be used to identify the most viable methods to mass-mark alewives for stock enhancement.

METHODS

Broodstock Acquisition

Sexually mature alewives were collected by the New Hampshire Fish and Game Department during their annual spawning migration from fish ladders located in the Lamprey

and Cocheco Rivers (Strafford County, New Hampshire) in May 2015. Alewives were distinguished from blueback herring by morphological differences (eye diameter and position, body depth; Loesch 1987) that were genetically validated previously (Berlinsky et al. 2015). Broodstock (total length [TL] ~250 mm, weight ~200 g) were transported to the Aquaculture Research Center at the University of New Hampshire (Durham, New Hampshire), and transferred to six 1,750 L tanks within an 11,000 L recirculating system equipped with biological and mechanical filtration, foam fractionation, ultraviolet sterilization, and supplemental aeration. Salinity and water temperature were maintained to simulate the natural spawning environment (0‰ and 19 – 20°C, respectively). During all handling procedures, broodstock were anesthetized with tricaine methanesulfonate (MS-222; 100 mg/L; Western Chemical, Ferndale, Washington) or clove oil (25-40 mg/L; Sigma-Aldrich, St. Louis, Missouri). Spawning and larviculture procedures were described previously (DiMaggio et al. 2015). Briefly, gender was determined by the presence of flowing eggs or milt upon application of gentle pressure, anterior of the urogenital opening. Females were implanted in the dorsal musculature with 95% cholesterol: 5% cellulose pellets (Sherwood et al. 1988) containing 25 µg [D-Ala^6 Des-Gly^{10}]-LHRH ethylamide (LHRHa; Bachem, Belmont, California). The spawned eggs were siphoned from tank bottoms, concentrated using a 500-µm mesh sieve, and quantified volumetrically. A subsample of 200 eggs from each spawn was evaluated under a Bausch & Lomb ASZ25L3 stereo microscope (Bausch & Lomb, Bridgewater, New Jersey) to determine developmental stage and fertilization success. Embryos were incubated in aerated, static MacDonald jars (21–24°C; 0‰ salinity) containing 400 mg/L formalin (37% formaldehyde; Parasite-S; Western Chemical, Ferndale, Washington) to prevent microbial contamination. The water in the hatching containers was completely replaced twice daily to prevent the accumulation of nitrogenous wastes from

decomposing eggs and hatched larvae. Eggs hatched in approximately 48-72 hours at 21-24°C. Alewife larvae (0-day post hatch [DPH]) were stocked into two static 40 L aquaria.

Oxytetracycline Immersion Marking

Six treatments compared three concentrations of oxytetracycline (200, 400 and 600 mg/L) for two marking durations (4 and 6 h). At 2 DPH, the larvae were condensed into a 5 L MacDonald hatching jar filled with 3 L of water and evenly dispersed with an air stone. Nine 1 mL samples were taken with a syringe to estimate population (~69,000 larvae). Six 40 L polyethylene conical tanks were filled with 30 L of water for marking (0‰ salinity, 17 mg/L $CaCO_3$ water hardness). Oxytetracycline hydrochloride (Pennox 343; Pharmgate Animal Health, Wilmington, North Carolina) was mixed with water to create solutions of 200, 400 and 600 mg/L. pH was maintained between 7.0 and 8.0 by adding equal parts potassium phosphate (dibasic) trihydrate (Acros Organics, Geel, Belgium) and sodium phosphate (dibasic) dihydrate (Sigma-Aldrich, St. Louis, Missouri) to the oxytetracycline mixture before being added to the treatment tanks. Larvae were evenly distributed into each tank and the tanks were covered with 3 mm black polyethylene sheets to prevent light exposure. Upon the conclusion of the treatments, the tanks were flushed with freshwater for one hour to reduce the concentration of oxytetracycline.

Larviculture

Following oxytetracycline marking, alewife larvae (3 DPH) were stocked into 12 round, black, 80 L polyethylene tanks (2 tanks/treatment) at densities of approximately 70 fish/L. Larvae for the thermal marking experiment were reared in two 700 L round polyethylene tanks

within a 4,700 L recirculating system until the beginning of the experiment. Tanks were gently aerated and held static from 0–45 DPH. Debris was siphoned from the tank bottoms daily, and 50% of the tank water volume was exchanged on alternate days to prevent the accumulation of nitrogenous wastes. Water temperature ranged from 19–21°C over the course of the trial and photoperiod was adjusted to simulate seasonal fluctuations (~13 h L: 11 h D; ~400 lx). Salinity was held at 5‰ using a mixture of fresh well water and synthetic sea salts (Instant Ocean; Spectrum Brands, Inc., Atlanta, Georgia). Larvae were fed rotifers, *Brachionus plicatilis,* enriched with an algal concentrate (Rotigrow Plus; Reed Mariculture, Campbell, California), three times daily at a feeding density of 10 rotifers/mL from 4–28 DPH. Rotifers were reintroduced when none or few remained in the water column. RotiGreen Nanno (*Nannocloropsis*; Reed Mariculture, Campbell, California) was also added to the larviculture tanks during rotifer feeding ("Green water"; >500,000 cells/mL) to increase larval feeding efficiency and preserve the nutrient profiles of the rotifers.

From 19-39 DPH, the larvae were co-fed decapsulated *Artemia nauplii* and rotifers, and when the majority of larvae were visibly consuming *Artemia,* rotifer feeding and microalgae supplementation were suspended. *Artemia* were fed two to three times daily at densities of 2 *Artemia*/mL from 19–39 DPH. Beginning 29 DPH the larvae were offered a commercially prepared 200-500 µm formulated diet (GEMMA Micro 300, Skretting, Stavanger, Norway), by coating the water surface layer (~600 µl portions). When the majority of larvae were visibly consuming the diet, *Artemia* supplementation was suspended, and the formulated diet was fed four times daily. The larvae were gradually transitioned to larger particle sizes (Otohime B2 (Reed Mariculture, Campbell, California), 360–650 µm, 51% crude protein, 11% crude fat and Gemma Wean Diamond (Skretting, Stavanger, Norway), 800 µm, 57% crude protein, 15% crude

fat) as larval growth progressed. At around 50 DPH, the oxytetracycline marked alewives were transferred to 235 L round polyethylene tanks (2 tanks/treatment) and reared for one year. Salinity was maintained at 5-10‰ and water temperatures ranged from 18-23°C throughout the year.

Mark Detection

To evaluate the presence of oxytetracycline marks, sagittal otoliths were extracted from six fish per treatment after 158, 232 and 350 DPH in October 2015 (5 months), January 2016 (8 months) and May 2016 (12 months). The otoliths were embedded sulcus side down on microscope slides with Loctite Gel Control Super Glue (Loctite, Düsseldorf, Germany) and sanded with 400, 1000 and 2000 grit sandpaper until the daily growth increments were exposed. The samples were stored in complete darkness to prevent photo degradation of the marks. The oxytetracycline marks were detected with a Zeiss Axioplan-2 Imaging Epifluorescence Microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a Lumen Dynamics X-Cite 120 (Excelitas Technologies, Waltham, Massachusetts) excitation light source. The otoliths were viewed under 200x magnification and a fluorescein isothiocyanate (FITC; 495-519 nm) filter cube was used to illuminate the mark. Images of the marks were taken using a Zeiss AxioCam MRm (Carl Zeiss AG, Oberkochen, Germany) monochromatic digital camera.

Thermal Marking – Larvae

Two treatments of thermal marking were conducted using two water temperature durations (12 h warm: 12 h cool, 24 h warm: 12 h cool) for two cycles. For each treatment, larval alewives (2 DPH) were distributed into three 1 L beakers filled with 0‰ water (~100

larvae/beaker) and manually transferred between two 150 L glass aquaria containing heated and cooled water (12 L) that served as static water baths. The water in the warm water bath was heated to 28°C with a Marineland Visi-Therm 100-Watt submersible aquarium heater (Marineland, Blacksburg, Virginia) and the water was chilled to 16°C in the cool water bath with a bench-top recirculating chiller fitted with titanium coils (PolyScience Series 9000 Refrigerated Constant Temperature Circulator, Niles, Illinois).

The experiment was repeated using the methods above but the larvae (2 DPH) were distributed into two 5L MacDonald jars filled with 3L 0‰ water (~100 larvae/jar). The warm water bath was heated to 24°C and the cold water bath was chilled to 18°C for two treatments of 12 h warm: 12 h cool and 24 h warm: 12 h cool. The experiment was again repeated using the above methods but the larvae were 13 DPH at the start. The larval thermal marking treatments experienced 100% mortality during the marking procedures.

Thermal Marking - Juveniles

Four treatments of thermal marking were conducted beginning at 44 DPH using two water temperature durations (12 h warm: 12 h cool, 24 h warm: 12 h cool) for two cycles. For each treatment, juvenile alewives were evenly distributed into four 38 L aquaria filled with 20 L of 0‰ water (~50 fish/tank) and manually transferred between thermally controlled 262 L recirculating water baths. The water in the warm water bath was heated with two Marineland Visi-Therm 100-Watt submersible aquarium heaters (Marineland, Blacksburg, Virginia) and the water was chilled in the cool water bath with a benchtop chiller fitted with titanium coils (VWR Scientific Model 1150A, Radnor, Pennsylvania). The four treatments were: 12 h at 25°C: 12 h at 18°C (44 DPH); 24 h at 25°C: 12 h at 18°C (46 DPH); 12 h at 28°C: 12 h at 16°C (51 DPH); and

24 h at 28°C: 12 h at 16°C (53 DPH). At the conclusion of the marking cycles, the juvenile alewives were stocked by treatment into 700 L round polyethylene tanks within a 4,700 L recirculating system and reared for one year.

Sagittal otoliths were extracted from six fish per treatment after 142, 271 and 349 DPH in October 2015 (5 months), February 2016 (9 months) and May 2016 (12 months) to evaluate the presence of the thermal marks. The otoliths were prepared using the methods described above and viewed under 100x magnification.

Statistical Analysis

A Pearson's chi-square (χ^2) goodness of fit test was performed using R version 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria) to determine if the percentage of alewives with visible oxytetracycline marks present was dependent on the duration of the immersion bath.

RESULTS

Oxytetracycline Marking

When viewed with an epifluorescence microscope, the oxytetracycline mark appeared as a fluorescent band around the core of the otolith (Figure 1). Higher concentrations of oxytetracycline resulted in a higher percentage of fish with visible marks. The percentage of marked fish was also influenced by the duration of the immersion bath ($\chi^2 = 50.27$; df = 5; $P < 0.05$). Larval marking with 200 mg/L exhibited low percentages of marked fish for both durations. The 400 mg/L treatments yielded 61 and 72% marked fish for the 4 and 6 h durations, respectively. The percentage of marked fish was high with the 600 mg/L 4 h treatment but

marks were not detected on one sample from October 2015. Marks were present on 100% of the otoliths sampled from the 600 mg/L 6 h treatment throughout the experiment (Table 1).

Thermal Marking

The width of the translucent increments on the otoliths was influenced by the amount of time the fish was exposed to the warm water bath. Uniform patterns formed on all otoliths sampled from each treatment. The 12 h warm: 12 h cold treatments produced evenly spaced increments that were indistinguishable from the daily growth increments. Wider increments formed when the fish were exposed to warm water for 24 h. Visually, there was no apparent difference between the lightness of the increments or darkness of the bands produced at 25°C: 18°C or 28°C: 16°C (Figure 2).

DISCUSSION

Oxytetracycline Immersion

Oxytetracycline produces marks on otoliths and other calcified structures. The chemical enters the bloodstream and rapidly binds to calcium ions during mineralization (Frost 1968; Campana 1999). The marks are permanently retained and are found in the growth increment that was being formed when the fish was exposed to the chemical (Campana 2001). Hard hatchery water (>150 mg/L $CaCO_3$) may negatively affect the uptake of oxytetracycline into calcified structures (Mauk 2008).

Fluorescent chemicals can be administered to fish for marking by feeding, injection or immersion. Incorporating oxytetracycline into formulated feed may take a week or more to produce a detectable mark on the otolith (Pedersen and Carlsen 1991). This method can also be

inconsistent because not all of the fish feed at the same rate (Hendricks et al. 1991; Pedersen and Carlsen 1991). Injecting individual fish is not feasible for marking large quantities of fish. It would also not be a feasible method for marking early life stages of alosines due to their sensitivity with handling stress. Immersion is the most viable technique for oxytetracycline marking because large quantities of hatchery-reared fish can be marked in several hours (Stewart and Long 2011).

The fluorescent marks degrade when exposed to direct sunlight (Doi and Stoskopf 2000). During the marking procedure, the tanks were covered with 3mm black polyethylene sheets to prevent photo degradation. After the otoliths were extracted and embedded in adhesive on microscope slides, they were stored in darkness to preserve the mark until it could be detected with the epifluorescence microscope. In a previous trial of oxytetracycline marking conducted in June 2014, the alewife larvae were marked in hard water (170 mg/L $CaCO_3$) and exposed to light during the marking procedure. The otoliths were extracted in October 2014 and only one mark, from the 600 mg/L for 6 h treatment, was detected.

Oxytetracycline marking is an effective method for producing long-lasting marks on the sagittal otoliths of hatchery-reared fish. It has been used to mark multiple species, including striped bass (*Morone saxatilis*), walleye (*Sander vitreus*), red drum (*Sciaenops ocellatus*), Allis shad (*Alosa alosa*) and American shad (*Alosa sapidissima*; Hendricks et al. 1991; Reinert et al. 1998; Jenkins et al. 2002; Logsdon et al. 2004; Lochet et al. 2009). In the present study, alewife larvae were successfully marked with oxytetracycline. Immersing the larvae in higher oxytetracycline concentrations for longer durations resulted in an increase in the percentage of fish with visible marks. Lochet et al. (2009) reported that mark intensity also increased when Allis shad larvae were exposed to higher concentrations for longer durations. The results of this

study suggest that immersion of larvae in concentrations of 600 mg/L for a duration of 6 h may be the most appropriate protocol for mass-marking alewives. This is higher than the optimal immersion concentrations suggested for American shad (200 mg/L) and Allis shad (300 mg/L; Hendricks et al. 1991; Jatteau 2010). Hendricks et al. (1991) immersed American shad larvae in concentrations of 25 and 50 mg/L for 12 h, and 200 and 400 mg/L for 6 h. The suggested optimal marking procedure for American shad was 200 mg/L for a duration of 6 h (Hendricks et al. 1991). Jatteau (2010) immersed Allis shad in 200, 250 and 300 mg/L for 4 and 6 h and suggested the optimal marking protocol to be 300 mg/L for 4 h.

Mark intensity was not evaluated in this study because it is dependent on background luminosity. Background luminosity is influenced by the thickness of the otolith, microscope slide and the adhesive used to affix the otolith to the slide (Lochet et al. 2009). The marks were easily identifiable and determining the presence and absence of marks was sufficient for this experiment.

Thermal Marking

Thermal marking is another technique for mass-marking fish. Otoliths continuously accrete layers of crystalline calcium carbonate forming daily growth increments (Brothers et al. 1976; Gauldie and Nelson 1990; Morales-Nin 2000). The patterns of translucent and opaque bands on the otolith can be influenced by altering the water temperature (Volk et al. 1999). Dense, opaque bands of otolin, a keratin-like protein, form in colder temperatures and higher temperatures form lighter, translucent increments composed of crystalline calcium carbonate between the bands (Letcher and Terrick 1998). Unique patterns can be created to differentiate between year classes and the hatchery the fish originated from (Volk et al. 1999).

In the present study, wider increments were deposited on the otoliths of fish exposed to warm temperatures for 24 h. This was consistent with thermal marking experiments conducted with Chinese suckers (*Myxocyprinus asiaticus*) and Atlantic salmon (*Salmo salar;* Letcher and Terrick 1998; Song et al. 2008). Evenly spaced increments were produced by the 12 h warm: 12 h cool treatments. These marks were indistinguishable from daily growth increments as the cycles of temperature changes were similar to natural diel cycles of otolith formation. Letcher and Terrick (1998) reported that larger temperature changes produced clearer, more distinct marks. In this study, there did not appear to be a difference in the lightness of the increments or darkness of the bands between the 25°C: 18°C (7°C temperature change) or 28°C: 16°C (12°C temperature change) treatments.

Thermal marking may not be an effective technique for mass-marking hatchery-reared alewives for stock enhancement. There are several disadvantages of stocking juvenile fish. Hatchery-reared fish are typically fed formulated pellets as raising live prey to feed to large quantities of fish in a hatchery is expensive and time consuming (Brown et al. 2003). Formulated pellets also contain essential nutrients to promote rapid growth and can be fed to fish with little effort (Brown et al. 2003). Foraging behavior develops with experience identifying prey sources, locations where prey can be found and how to consume the prey (Brown and Laland 2001; Brown et al. 2003; Warburton 2003). Hatchery-reared fish that were released into the wild have been found to consume objects that resemble formulated pellets, including detritus, shells and stones (O'Grady 1983; Ellis et al. 2002).

Predation is one of major causes of mortality among hatchery-reared juvenile fish shortly after release into the wild (Olla et al. 1998). Anti-predator behaviors develop with experience. Hatchery-reared fish may not be able to recognize predatory species or know how to evade them

(Olla et al. 1998; Vilhunan 2006). The stress of handling and transportation may also make the fish susceptible to predation shortly after stocking (Olla et al. 1998).

There are advantages of marking larvae for stock enhancement. Large quantities of larvae can be marked and released before they begin feeding so they can develop foraging behaviors in the natural environment. They will also be able to develop anti-predator behaviors in the wild because they did not become acclimated to the hatchery environment. However, thermal marking was not feasible with alewife larvae because they were unable to withstand the temperature changes and all of the treatments experienced 100% mortality.

Conclusions

Biologists can collect a wide variety of information from mark and recapture studies. Marking alewives can be used to evaluate natal homing and the contribution of hatchery-reared fish to wild populations. Studies have been conducted marking American shad with oxytetracycline. In the Lehigh River in Pennsylvania, marks were detected on 73-98% of the returning American shad sampled between 1995 and 2000 (Hendricks et al. 2002). Catch rates in Virginia's James River increased in 2000 and 2001 due to an influx of sexually mature hatchery-reared shad. Marks were detected on over 40% of the harvested shad (Olney et al. 2003). Marking otoliths can also be used for age validation. By marking otoliths at a known age, the deposition rate of the increments and rings can be validated (Geffen 1992).

In conclusion, oxytetracycline immersion is an effective method for marking alewife larvae. Based on this study, the recommended protocol for marking alewives is a six-hour immersion in a concentration of 600 mg/L. Thermal marking may not be an effective method for mass-marking alewives. The lack of foraging and anti-predator behaviors in hatchery-reared

juvenile fish typically result in high mortality rates shortly after stocking. Alewife larvae were also unable to withstand the temperature changes of the marking procedures resulting in 100% mortality. The results from this study will benefit future mark and recapture research with alewives.

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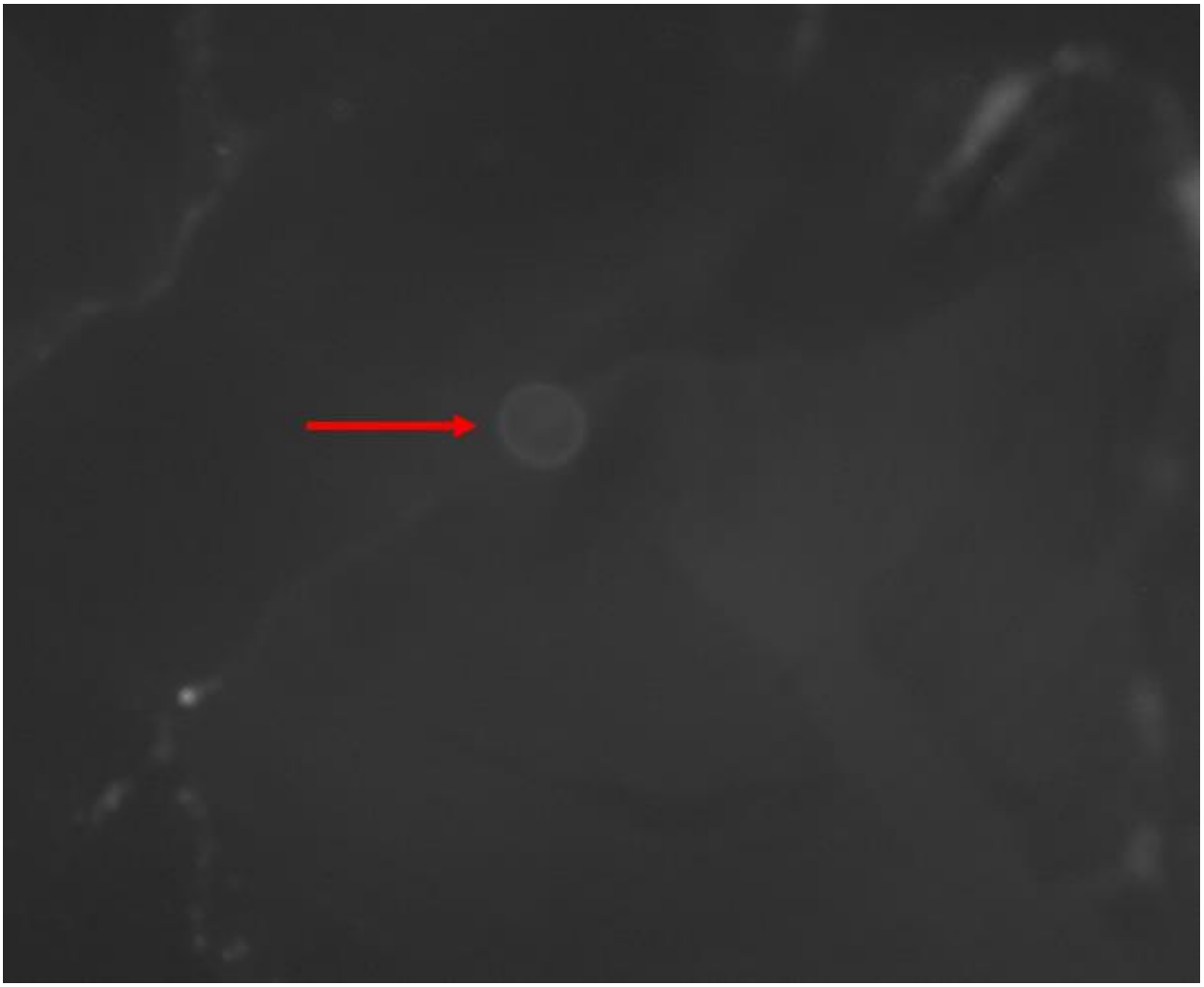

Figure 1. Oxytetracycline mark examined 12 months after immersion in 600 mg/L for 6 h. The otolith was viewed under 200x magnification using an epifluorescence microscope.

Table 1. Percentage of alewives with visible marks from the oxytetracycline immersion treatments. Sagittal otoliths were sampled from 6 fish per treatment at 5 months (October 2015), 8 months (January 2016) and 12 months (May 2016).

Treatment	**5 Months (%) n = 6**	**8 Months (%) n = 6**	**12 Months (%) n = 6**	**Total (%)**
200 mg/L 4 h	0.0	33.3	16.7	16.7
200 mg/L 6 h	16.7	16.7	16.7	16.7
400 mg/L 4 h	50.0	66.7	66.7	61.1
400 mg/L 6 h	66.7	83.3	66.7	72.2
600 mg/L 4 h	83.3	100.0	100.0	94.4
600 mg/L 6 h	100.0	100.0	100.0	100.0

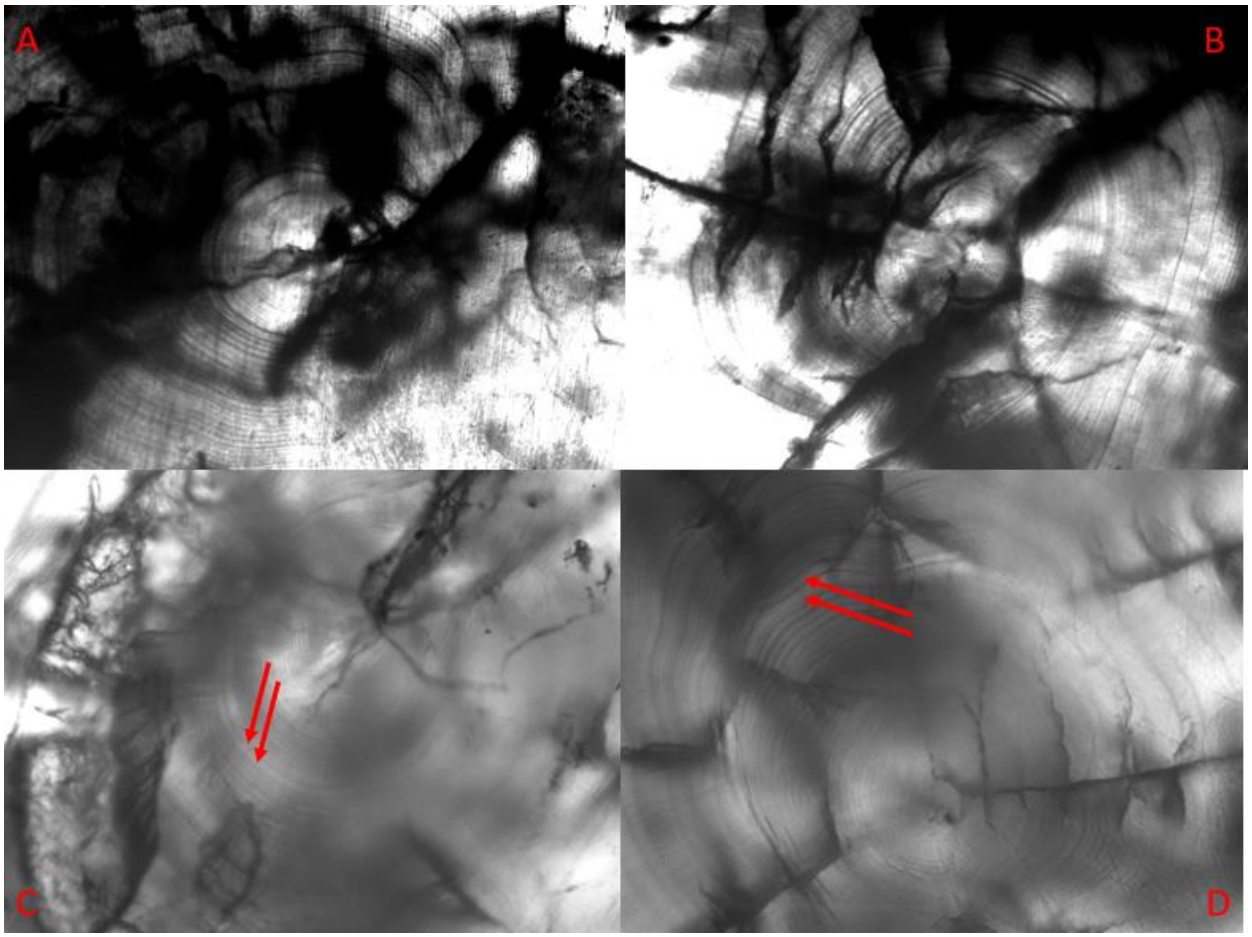

Figure 2. Thermal marks on the sagittal otoliths of alewives sampled in October 2015 viewed under 100x magnification.
A. 12 h at 25°C: 12 h at 18°C
B. 12 h at 28°C: 12 h at 16°C
C. 24 h at 25°C: 12 h at 18°C
D. 24 h at 28°C: 12 h at 16°C

CHAPTER 3

CONCLUSIONS

In summary, oxytetracycline immersion is an effective method for mass-marking hatchery-reared alewives. The percentage of fish with visible marks present increased in treatments with higher concentrations for longer immersion durations. The present study indicates immersion of larvae into a concentration of 600 mg/L for 6 h is the optimal method for marking alewives.

The larvae in the thermal marking experiment were stressed by the temperature changes and experienced 100% mortality during the marking procedures. None of the juveniles died during thermal marking. Uniform patterns were present on all of the otoliths sampled. Exposure to warm water for 24 h created wider translucent increments between the bands on the otolith. Exposure to warm water for 12 h then cool water for 12 h produced increments of equal width that were indistinguishable from the daily growth increments. There did not appear to be a difference between the lightness of the increments or darkness of the bands in the thermal marks produced by the 25°C: 18°C (7°C temperature change) or 28°C: 16°C (12 °C temperature change) treatments.

Marking alewives can be used to collect a wide variety of information. Mark and release studies can be used to evaluate natal homing in alewives. They can also be used to measure the success of stock enhancement programs. In studies marking American shad with oxytetracycline, hatchery-reared fish have significantly contributed to the spawning populations in several rivers (Hendricks et al. 2002; Olney et al. 2003). Age validation can also be evaluated

by otolith marking. This can be used to validate age at maturity, lifespan and estimate growth rates. Fisheries managers can use this data to develop age-based stock assessment models, and to estimate natural mortality and population structure. The results from this study will benefit future marking studies with alewives.

Future studies should be conducted to develop oxytetracycline marking protocols for blueback herring. Thermal marking studies with river herring larvae should also be revisited to find temperature ranges that will produce detectible marks with low mortality.

APPENDIX A. ANIMAL CARE AND USE APPROVAL DOCUMENTATION

University *of* New Hampshire

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51 College Road, Durham, NH 03824-3585
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21-Mar-2013

Berlinsky, David L
Biological Sciences, Rudman Hall
Durham, NH 03824

IACUC #: 120404
Project: River Herring Aquaculture and Population Assessment
Category: D
Next Review Date: 25-Apr-2014

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your request for a time extension for this protocol. Approval is granted until the "Next Review Date" indicated above. You will be asked to submit a report with regard to the involvement of animals in this study before that date. If your study is still active, you may apply for extension of IACUC approval through this office.

The appropriate use and care of animals in your study is an ongoing process for which you hold primary responsibility. Changes in your protocol must be submitted to the IACUC for review and approval prior to their implementation.

Please Note:
1. All cage, pen, or other animal identification records must include your IACUC # listed above.
2. Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. Participation is mandatory for all principal investigators and their affiliated personnel, employees of the University and students alike. Information about the program, including forms, is available at http://unh.edu/research/occupational-health-program-animal-handlers.

If you have any questions, please contact either Dean Elder at 862-4629 or Julie Simpson at 862-2003.

For the IACUC,

Jill A. McGaughy, Ph.D.
Chair

cc: File